

# Asymbiotic Seed Germination in Orchids: Role of Organic Additives

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**Abstract:** Orchid seeds are unique in being exceedingly small, dust like in appearance, and more or less fusiform in shape; these lack endosperm and have undifferentiated embryos enclosed within transparent seed coats. Their germination in nature is dependent upon a suitable association with a mycorrhizal fungus. Their fungal requirement can, however, be compensated by supply of sugars and other mineral nutrients in vitro, and several orchid species from diverse habits and habitats have successfully responded to asymbiotic germination, much, however, still remains to be learnt about the nutrient requirements of commercially important and/or endangered orchid species, keeping in view of the large size of the orchid family. This paper attempts to review the available literature on various aspects of asymbiotic seed germination in orchids and the role of organic growth additives in promoting seed germination, protocorm development and growth of seedlings.

**Keywords:** Orchids, asymbiotic seed germination, in vitro culture, organic additives.

## I. INTRODUCTION

The Orchidaceae, comprising 30,000–35,000 species belonging to 850 genera, is the largest, highly evolved and diverse family of flowering plants, and accounts for 10% of flowering plants or almost 30% of monocotyledons (Dressler, 1993; Lucksom, 2007). About 70% of orchids are epiphytes and comprise almost two-third of the world's epiphytic flora (Gravendeel et al., 2004). Of the rest, 25% are terrestrial and 5% can be found on various supports (Atwood, 1986). The orchids are well known for their exquisite and long-lasting flowers which have made them doyen among ornamentals. They are characterized by a highly specialized pollination system, small, thin and non-endospermic seeds, obligate requirement of mycorrhizal association during germination, diverse and cosmopolitan habitats, advanced evolutionary trends, extraordinary mechanisms of adaptation and persistence in adverse environmental conditions (Rasmussen, 1995; Arditti, 1992; Phillips et al., 2009). Consequently, they have always fascinated botanists, horticulturists, and evolutionary biologists alike.

The orchids are inherently slow growers; their growth and development are markedly influenced by specialized microclimatic conditions and protective canopy of the floristics in their natural habitats (Vij, 1995). In the recent years, wanton clearance of forests for developmental and agricultural purposes has jeopardized the existence of the natural habitats and populations of a large number of orchids. Unregulated collection of orchids for commercial and herbal purposes has compounded the problem further. As a result, the majority of orchids have become an object of concern to conservationists due to its high sensitivity to alterations in its environment. In situ conservation by preservation and enhancement of dwindling populations of endangered orchid species is very difficult because of the relatively slow growth of orchids and low germination rates which requires symbiotic relationships with mycorrhizal

fungi in natural habitats (Rasmussen, 1995). Consequently, in vitro mass propagation techniques are being increasingly utilized for conservation and commercialization of orchid species and hybrids. Ever since the development of a protocol for asymbiotic seed germination of orchids by Knudson (1922), the technique has become an important and favored method for propagation of a variety of terrestrial and epiphytic orchid species (Yamazaki and Miyoshi 2006; Stewart and Kane 2006).

## II. ORCHID SEEDS

The structure and size of the seed are among the most striking characteristics of orchids. Orchid seeds are very small (0.05-6.0 mm in length and 0.01-0.9 mm in diameter), extremely light (0.31-24  $\mu$ g), and produced in large number (50-4,000,000 per capsule) (Arditti and Ghani, 2000). The seed, which may at times be apomictic (Stoutamire, 1964), has been compared to fern spores (Went, 1949) and consists of a small spherical embryo suspended within a membranous, often transparent, but at times pigmented, seed coat (Arditti, 1967). In most orchids, the embryo is undifferentiated and the endosperm development is suppressed (Cocucci and Jensen, 1969). While, the mature embryo of *Epipogium aphyllum* always comprises 8 cells, the number of cells in the mature embryo, in a majority of species, has been reported to range from 29 (*Calypso bulbosa*; Harvais, 1974) to ca. 200 (*Dactylorhiza majalis*; Rasmussen, 1990). *Bletilla striata*, considered to possess one of the most highly developed embryos, comprises about 734 cells (Rasmussen, 1995). The orchid seeds at maturity contain lipidaceous food reserves, which occur as discrete inclusions within the cells of the embryo (Poddubnaya-Arnoldi and Zinger, 1961). Analysis of *Cymbidium* seeds showed that they contain 32% lipids, 1% sugars, and no starch (Knudson, 1929), although starch is reported in the embryos of other orchid

species (Arditti and Ernst, 1981). Because of the limited food reserves in the minute seeds, orchids in their natural habitats go through a heterotrophic phase in which the developing plants rely on a balanced relationship with fungal endophytes for nutrition, and this reliance begins at the onset of germination (Smreciu and Currah, 1989). Since the uninfected orchid seedlings are unable to grow on polysaccharides and attempts to germinate seeds asymbiotically in the absence of simple sugars are invariably unsuccessful, even with the addition of vitamins, hormones, or other substances (Arditti and Ernst, 1981), the need for fungal infection of orchid seeds appears to be due to an impaired ability of the seeds to metabolize polysaccharides and lipids (Manning and van Staden, 1987). The fungus is believed to provide the necessary germination stimulus by aiding carbohydrate, nitrogen, mineral, and vitamin transport during germination (c.f. Arditti, 1967).

### III. IMMATURE SEED GERMINATION

Ever since Knudson (1922) demonstrated that the fungal requirement of orchid seeds can be successfully bypassed in vitro using a relatively simple culture medium containing sucrose, asymbiotic seed germination has emerged as an important procedure for propagating a large number of orchid species and hybrids representing diverse habits and habitats (Arditti et al., 1982; Miyoshi and Mii, 1995, 1998; Pathak et al., 2001; Piri et al., 2012). The orchid seeds are also capable of germination, in vitro, prior to reaching maturity, and their culture often referred to as "ovule/embryo/green-pod" culture (Sagawa, 1963) has added new dimensions to conservation and commercialization of orchids.

The technique has been positively tested in a large number of taxa (Arditti et al., 1982; Vij and Pathak, 1988; Yam and Weatherhead, 1988; Shiao et al., 2002). The technique involves an easy procedure of sterilization, ensures better frequency of germination, reduces the time lapse between pollination and sowing of seeds, and helps in i) production of virus-free seedlings, ii) propagation of rare and endangered species, and iii) recovering progenies of desired matings (Vij et al., 2000). Its additional utility in exploiting the polyembryonate potential of orchid seeds and cloning their apomictic (obligate) genotypes has also been highlighted in several ground orchids which often tend to bypass sexuality in favour of apomixis (Vij, 1995).

The better germination potential of immature seeds has been attributed to their distended testa cells and metabolically awakened embryos besides lack of dormancy and/or inhibitory factors (Linden, 1980; Yam and Weatherhead, 1988). The desiccation that takes place in the intact maturing fruit is interrupted when ovules are excised and transferred to a culture medium with low osmolarity. Since declining water potential is probably a regulatory factor for protein accumulation in ripening seeds, their rehydration during germination in vitro generates a developmental change from protein storage to protein mobilization (Kermode, 1990). However, as all the

seeds/embryos are used in a single sowing in the "green-pod" culture technique, the importance of a proper stage at which the capsule has to be harvested in a particular taxon assumes great significance. The immature seeds are often excised while the embryo cells are still fully hydrated and mitotically active. Bhojwani and Razdan (1996) reported that the nutritional requirements of the young embryos are complex as compared to the mature ones. According to Arditti et al. (1982), the seeds collected from capsules after about half of the time they take to mature show a better germination response. Based on a comparative analysis in 47 species and hybrids, Nagashima (1989) hinted at a strong correlation between periods from pollination to fertilization and from pollination to the completion of embryogenesis, and also between the periods from pollination to fertilization and from pollination to the stage of maximum germinability.

### IV. MATURE SEED GERMINATION

The mature seeds, on the other hand, often germinate with difficulty due to change in the quality of their food reserves. Harvais (1974) reported that food reserves comprise starch in immature, and lipids in the mature seeds of *Corallorhiza maculata* and suggested that conversion of starch and other simpler carbohydrates into lipids during seed maturation may be a common feature of orchids. The inability of mature seeds to germinate with ease has also been attributed to the lack of an appropriate metabolic machinery (glyoxysomes) capable of utilizing their own lipidaceous reserves (Harrison, 1977); accumulation of germination inhibitors in the seed coat; onset of dormancy in the mature seed; and loss of viability (c.f. De Pauw and Remphrey, 1993). Since dormancy of mature seeds in orchids has been successfully broken by treating these with cold temperature (Harvais, 1974) or sucrose (Weatherhead et al., 1986), it appears that dormancy rather than the non-viability is responsible for poor response of the mature seeds in vitro.

In *Dactylorhiza maculata*, the abscisic acid contents which are 15 times more in the mature seeds as compared to the immature ones, may be responsible for seed dormancy (Van der Kinderen, 1987). Raghavan (1976) recorded changes in the enzyme complements at different stages of seed maturation in orchids, but the critical stage at which they acquire dormancy, is yet to be identified. Moreover, as the dormant (mature) and metabolically active (immature) seeds are morphologically more or less indistinct, Mitra (1986) stressed the importance of information on histochemical and biochemical features for selecting the right type of immature ovules (seeds).

The epiphytic species germinate better than the terrestrial ones due probably to their simpler nutritional requirements (Arditti et al., 1982), and the present species were no exception. The stringent nutritional requirements of the terrestrials for germination in vitro have been attributed to their greater mycorrhizal needs (Stoutamire, 1974). In this connection, it is worthwhile to mention that in vitro requirements of seeds/embryos are greatly influenced by the level of their maturity and genetic and ecological amplitudes of the species (Anderson, 1991).

## V. EFFECT OF ORGANIC ADDITIVES

Growth and morphogenesis of plant tissues can be promoted by the addition of various organic supplements and plant extracts (Fonnesbech 1972). A large and bewildering number of organic additives, unwanted or untried in other plants, are routinely employed to enrich the culture media for orchid seed germination. Some such common as well as uncommon additives are apple juice, banana homogenate, beef extract, casein hydrolysate, coconut water, extract of silkworm pupae, fish extract, honey, peptone, potato extract, tomato juice, and yeast extract, although they have undefined mixture of organic nutrients and growth factors (Islam et al. 2003; Murdad et al. 2010).

### Peptone

A water soluble protein hydrolysate with high amino acid content, Peptone (P) supported growth of *P. spicerianum* cultures (Flamee, 1978). A perusal of literature reveals that P was obligatory for germination in *Spiranthes cernua* (Stoutamire, 1974) and for inducing organogenesis in *Goodyera biflora* (Pathak et al., 1992). It improved germination [*Dactylorhiza* (Linden, 1980)], supported seedling growth [*Paphiopedilum*, *Phaius*, *Vanda* (Curtis, 1947), *Geodorum densiflorum* (Roy and Banerjee, 2001)], and induced protocorm multiplication [*Cymbidium macrorhizon* (Vij and Pathak, 1988)]. Growth promotion of protocorms by P has been reported for orchid species like *Spathoglottis plicata* (Curtis, 1947), *Epidendrum ibaguense* (Hossain, 2008), and *Calopogon tuberosus* (Kauth et al. 2006). However, P impaired germination in *Dactylorhiza maculata* (Van Waes and Debergh, 1986) and *Vanda tessellata* (Roy and Banerjee, 2002), and proved ineffective in *Calanthe discolor* (Kano, 1965) cultures. All these data suggest that efficacy of P varies with the species and the developmental stage of the germinating entities. According to Arditti et al. (1982), the discordant effect may also be attributed to the source and batch of P supply.

### Coconut Water

Coconut water (CW) is the colorless liquid endosperm of green coconuts (*Cocos nucifera* L.), which contain soluble sugars as a natural source of carbon, amino acids, phenols, fiber and vitamins, moreover, it also contains diphenyl urea which functions as cytokinin that can enhance the explant growth and regeneration by inducing cell division (Gnasekaran et al., 2010; Texeira da Silva et al., 2006). For years, simple supplementation of nutrient media with CW has remained a standard procedure to obtain satisfactory growth and organ differentiation in orchid cultures. CW induces division of the otherwise non-dividing cells (George and Sherrington, 1984) and mass multiplication of protocorms (Sagawa and Kunisaki, 1984) in orchids. The concentration of CW employed for this purpose ranges from 10-30% (Sagawa and Kunisaki, 1984). The growth promotory effect of CW has been attributed to its PGR content, and the most important PGRs are the cytokinins (Latham, 1974; van Staden and Drewes, 1975), whereas auxin and gibberellin-like constituents have also been reported (Dix and van Staden, 1982). Huang and Hu (2001)

reported that 5% CW, banana homogenate or potato homogenate in Harvais medium could accelerate the seed germination of *Cypripedium flavum*. Similar results have been reported in different plant species. For instance, the fresh and dry biomass of *Anoectochilus formosanus* increased significantly when 50 ml/L CW and 0.5 g/L activated charcoal were added during bioreactor culture (Yoon et al., 2007). Hyponex medium supplemented with 50 ml/L CW enhanced fresh and dry biomass, number of roots, leaf area as well as development of healthy plantlets of *Calanthe* hybrids (Baque et al., 2011). Similarly, 10% CW promoted the proliferation of protocorm-like bodies of *Dendrobium Alya Pink* (Nambiar et al., 2012).

Recently, Huh et al. (2016) analyzed the constituents of CW and reported that it contained around 2.5% sugars and sucrose was the main sugar. CW also contains variable inorganic ions such as potassium, phosphorus, calcium, magnesium, iron and manganese. Water-soluble vitamins such as thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), myo-inositol and ascorbic acid (C), which were recommended for orchid growth, were also detected in CW. Coconut water also contained organic acids including citric acid, malic acid and succinic acid. Phytohormones such as Indoleacetic acid, trans-Zeatin riboside and Abscisic acid were also detected in CW.

### Banana Homogenate

Graefflinger (1950; cited in Ernst et al., 1970) first proposed the use of banana fruit powder for the germination of orchids. Withner (1953) observed beneficial role of mashed banana or banana homogenate (BH) in the germination of *Paphiopedilum* seeds. Ernst (1982) recorded a growth promotory effect of BH on *Paphiopedilum* seedlings. An analysis of banana fruit pulp revealed that it contains different carbohydrates, minerals, amino acids, fatty acids, niacin, vitamins, cellulose, polyols, and sterols (Tamura, 1970). The stimulatory effect of BH on seed germination and seedling development has also been observed in *Anoectochilus formosanus* (Shiau et al., 2002), *Cattleya* (Islam et al., 2000), *Hetaeria cristata* (Yam and Weatherhead, 1990), and *Phalaenopsis* (Ernst, 1986). In studies with *Paphiopedilum ciliolare*, Pierik et al. (1988) reported that the effect of BH was inhibitory during seed germination and beneficial only for further development of seedlings.

### Yeast Extract

Yeast Extract (YE) is also an important source of organic nitrogen (amino acids) and has been effectively used in germination and proliferation in many orchid species (Mitra, 1986).

Studies conducted in terrestrial orchid seeds and protocorms revealed more efficient utilization of amino acids by young protocorms (Curtis 1947; Curtis and Spoerl 1948; Malmgren 1996). Greater preference for nitrogen from amino acids rather than from ammonium or nitrate salts has also been favored for some epiphytic orchids (Nadarajan et al. 2011).



### Casein Hydrolysate

Casein hydrolysate (CH) is an amino acid complex and has been widely used in orchid seed germination media. Beneficial role of CH in seed germination and seedling growth has been demonstrated in *Dactylorhiza purpurella* (Harvais, 1972), *Aerides multiflora*, *Rhynchostylis retusa*, *Saccolabium papillosum*, *Vanda testacea* (Vij et al., 1981), and *Bletia urbana* (Rubluo et al., 1989). Decruse et al. (2013) have also reported enhanced growth of protocorms of *Eulophia cullenii* in medium supplemented with CH. However, CH was reported to impair germination in *Herminium lanceum* and seedling development in *Coelogyne barbata* (Chand, 1991). Growth promotory effect of CH was not pronounced in *Dactylorhiza maculata* (Van Waes and Debergh, 1986) and *Vanda cv. Miss Joaquim* (Rao and Avadhani, 1964) cultures. The variable effect of CH during germination may be attributed to its composition, which varies with extent of hydrolysis of the nitrogenous substances present in the casein (Arditti et al., 1982).

### VI. CONCLUSION

Asymbiotic seed germination provides an efficient way for mass propagation of orchids. Development of efficient seed germination and acclimatization protocols that focus on propagating orchid seedlings for reintroduction in their natural habitats will help in the conservation of endangered or threatened orchid species. Addition of organic additives to orchid seed germination medium promotes seed germination, accelerates protocorm formation and produces vigorous seedling in a majority of the reports in literature. These organic additives provide a natural source of carbohydrates, inorganic ions, amino acids, vitamins and phytohormones, and help in orchid propagation by promoting growth and morphogenesis in asymbiotic seed cultures.

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